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PRINCIPAL INVESTIGATOR: Felix W. Wehrli, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104

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14. ABSTRACT

Assessment of axon health in spinal cord injury (SCI) is vital for proper diagnosis and treatment. Magnetic resonance imaging (MRI) is routinely performed in patients and provides valuable information about cord edema and hemorrhage. However, comprehensive prediction of axonal changes from in vivo MR imaging remains elusive. At the U. Penn site, we are applying two novel MRI methods to the problem of assessment of axonal loss, axonal diameter distribution, and myelin loss (q-space imaging (QSI) and ultra-short echo-time (UTE) MRI) first on animal specimens and then on human subjects.

During the reporting period we have further developed and published the UTE MRI method for myelin quantification. Our results suggest that UTE MRI will be able to quantify myelin content. Direct quantification of myelin content would remove ambiguities that exist in indirect methods leading to a more accurate assessment of myelin health. We have all injured and perfusion fixed spinal cords representing the different post-injury periods in our possession (supplied by the Drexel performance site). However, we have not been able to execute the QSI due to problems related to the scanner upgrade involving matching of our custom gradients to the instrument (detailed in the report below). However, we anticipate resumption of scanning before 12/12 and completion of the work in 2013.

15. SUBJECT TERMS

Axon Architecture, Spinal Cord Injury, Axon Loss, Myelin, Q-Space Imaging, UTE, MRI

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INTRODUCTION

Spinal cord injuries (SCI) produce direct mechanical disruption with subsequent severe degeneration of axons, and are the processes underlying the associated neurologic deficits observed in such injuries. Histological studies of fixed tissue in animal models of SCI have described axonal loss and demyelination occurring after trauma. Research at the University of Pennsylvania site brings novel magnetic resonance methodology to bear with the objective of obtaining quantitative information on axonal degeneration and myelin loss following spinal cord injury in a mouse model by pursuing the following Specific Aims per the work statement:

1. *We will perform q-space MR imaging (QSI) and simulations of QSI to quantify axonal architecture in healthy and injured mouse spinal cords.*
2. *We will quantify myelin content with three quantitative MRI techniques in healthy and injured mouse spinal cords and compare the results with histology.*

Specific Aim 1:

Subsequent to the upgrade of the Bruker NMR/MRI system, hardware modifications were needed to connect our previous custom gradient coil to the new system. The gradient coil also had to be optimized and recalibrated for the new system. This included a systematic and thorough analysis of RF noise sources in the hardware, resulting in the implementation of new shielding and a new filter box on the gradient leads to isolate the receive chain from both room-ambient RF signals and RF noise leakage from the gradient amplifier. Our implementations were similar in design to those used by Bruker for their gradient coils. In addition, we discovered a crack in the epoxy of our custom gradient coil that produced a vibrational phase instability in the data, and we corrected this by applying a new layer of epoxy resin to the gradient coil windings. After these modifications to the gradient coil hardware had been accomplished, we performed a new calibration of our custom gradient coil as this was needed to accurately calculate axon diameters from the q-space data. To ensure a reliable calibration we used three independent methods that agreed with each other: 1) a high-resolution transverse image of a capillary tube of known inner diameter (measured by micro-CT) filled with PEG in water was acquired using our custom gradient for the frequency axis. The Bruker gradient for the phase axis and the frequency-axis scale factor was adjusted to give equal frequency/phase tube diameters; 2) projections of the same PEG/water tube were acquired orthogonal to the tube at varying gradient amplitudes using our custom coil and the resulting NMR line widths were plotted versus actual current applied to the coil; and 3) the ADC of deionized water was measured using our custom coil and compared to the literature value at the same temperature. Finally, as the old QSI pulse sequence program did not run on the new system, a new QSI pulse sequence program had to be developed.

Once the new pulse sequence program has been tested, QSI experiments will be performed, followed by histologic analysis and QSI simulations.

Progress has been made toward translation of the QSI methodology to the clinic (as reported in part previously). The pulse sequence designed toward the end of the prior reporting year has since yielded QSI displacement maps on 1.5T on a clinical imager (Siemens Sonata) on fixed pig spinal cords. Since the pig spinal cord is similar in size to the human spinal cord and the experiments were performed with standard imaging gradients the results suggest feasibility of performing studies in humans with spinal cord injury (even though this was not a specific objective of the current project). This research was presented as a poster at the 2012 Annual Meeting of the International Society for Magnetic Resonance in Medicine in Melbourne, Australia. Notably, we have been able to distinguish white matter tracts differing in axon diameter and density. Figure 1a shows analyzed white matter regions. Figure 1b shows the mean displacements in the three regions, commensurate with the different axon sizes (1).

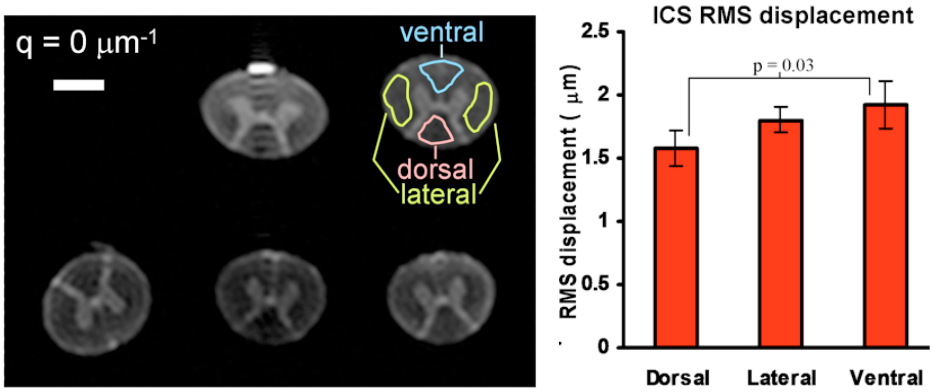

Figure 1 a) Sample transverse images of pig spinal cord acquired at 1.5T with regions of interest indicated (white bar = 5mm); b) RMS displacement obtained from QSI displacement maps consistent with known axon diameters in the three WM tracts examined.

Specific Aim 2:

We previously reported preliminary results on our endeavors toward direct imaging of myelin in the spinal cord of rodents by 3D ultra-short echo-time (UTE) MRI. This work has now been published in the Proceedings of the National Academy of Sciences (PNAS) (2). In brief: We identified the spectrum of myelin in the spinal cord *in situ* as well as in myelin lipids extracted via a sucrose gradient method, and investigated its spectral properties. High-resolution solution NMR spectroscopy showed the extract composition to be in agreement with myelin's known chemical make-up. The 400-MHz ^{1}H spectrum of the myelin extract, at 20ºC (room temperature) and 37ºC, consists of a narrow water resonance superimposed on a broad envelope shifted approximately 3.5 ppm upfield, suggestive of long-chain methylene protons. Superimposed on this signal are narrow components resulting from functional groups matching the chemical shifts of the constituents making up myelin lipids. The spectrum could be modeled as a sum of super-Lorentzians with a T2* distribution covering a wide range of values (0.008-26 ms). Overall, there was a high degree of similarity between the spectral properties of extracted myelin lipids and those found in neural tissue. The normalized difference spectrum had the hallmarks of membrane proteins not present in the myelin extract. Using 3D radially ramp-sampled proton MRI, with a combination of adiabatic inversion and echo subtraction, the feasibility of direct myelin imaging in situ is demonstrated. Last, the integrated signal from myelin suspensions is shown, both spectroscopically and by imaging, to scale with concentration, suggesting the potential for quantitative determination of myelin density in both healthy and injured spinal cords.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated feasibility of translation of the Q-Space mapping technique of axon diameter, intra- and extra-axonal space, from the laboratory to the clinic.
- Isolated myelin and showed that its NMR properties match those of myelin in situ in the spinal cord.
- Demonstrated a path toward quantitative myelin imaging in humans.
- Identified the magnetic resonance signal of myelin and showed feasibility of direct imaging of neural myelin as a new metric for the evaluation of spinal cord injury.
- Fully characterized the relaxation properties of myelin toward a more complete understanding of the MRI signal in quantitative myelin imaging (Wilhelm et al, 20th Annual ISMRM Scientific Meeting, Melbourne, Australia (3))
- Published major paper in the Proceedings of the National Academy of Sciences (Wilhelm, et al, PNAS 2012).

- Performed experiments on the upgraded Bruker Instruments micro-imaging system after interfacing custom-built gradients for high-resolution q-space imaging and achieved performance goals.

OUTCOMES

The new myelin imaging technique developed under this grant has shown feasibility for quantitative assessment of myelin content in the CNS of the injured rat spinal cord.

CONCLUSION

While substantial progress has been made toward achievement of the goals, the main objective to obtain data in the injured cord and the hypothesized temporal changes has not been attained yet, largely due to the technical problems that have now been solved. Our partners at Drexel University have provided the injured, fixed spinal cords and we are confident we will complete the project during year 3 of this award.

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Feasibility of low q-space diffusion MRI at 1.5T

Henry H. Ong[1], Yusuf Bhagat[1], Jeremy Magland[1], and Felix W. Wehrli[1]

[1]*Laboratory for Structural NMR Imaging, Departement of Radiology, University of Pennsylvania School of Medicine, Philadelphia, PA, United States*

Introduction

By exploiting the regularity of molecular diffusion restrictions such as axon membranes and myelin sheaths[1], q-space imaging[2,3] (QSI) offers potential for indirect assessment of white matter (WM) axonal architecture. For example, QSI can accurately estimate mean axon diameter (MAD) and intracellular volume fraction (ICF)[4,5]. Unfortunately, the application of QSI on a clinical scanner is severely constrained by the low gradient strengths available, which limits the maximum achievable q-value ($q = (2\pi)^{-1}\gamma G\delta$, G = gradient amplitude, and δ = gradient duration). Low maximum q-value leads to insufficient displacement resolution to accurately study axons, which have an MAD of 1-3 µm. Low q-value diffusion MRI[5], in which axonal architecture information is extracted by fitting the q-space signal decay (E(q)) at low q-values (q^{-1} >> MAD) under the short gradient pulse approximation (SGPA), does not require high gradient amplitudes. However, low clinical gradient strengths lead to violation of SGPA. Here, we test the feasibility of implementing low q-value diffusion MRI on a 1.5T system by assessing axonal architecture in excised fixed pig spinal cords.

Methods

As described in [5], at low q-values (q^{-1} >> MAD), the signal decay is given by $E(q)=\exp(-2\pi^2q^2Z^2)$ (Eq.1), where Z is the root mean squared (RMS) displacement of diffusing molecules during a diffusion time Δ[6,7]. As E(q) contains signal from extra- and intra-cellular spaces (ECS and ICS), a two-compartment version of Eq.1 can be defined: $E(q)= f_E\cdot\exp(-2\pi^2q^2Z_E^2) + f_I\cdot\exp(-2\pi^2q^2Z_I^2)$ (Eq. 2), where f_E and f_I are the relaxation-weighted ECS and ICS volume fractions and Z_E and Z_I are the RMS displacement of diffusing molecules in the ECS and ICS. From Eq. 2, MAD and ICF can be estimated from Z_I and f_I, respectively.

For validation of this method, five fixed cervical spinal cords (SC) harvested from five skeletally mature Yucatan mini-pigs were used. Before experiments, the SCs were placed in tubes filled with Fomblin (Sigma-Aldrich) to keep the specimens hydrated and to remove any background signal. The low q-value diffusion MRI method was implemented on a 1.5T Siemens Sonata MRI scanner (Erlangen, Germany) with 40 mT/m gradients using a custom single-slice PGSE with multi-shot fly-back EPI readout pulse sequence. The body coil was used for transmit and a custom-built 4-channel phased array coil (Insight MRI) was used for receive. The imaging parameters were: Δ/δ/TE = 98.7/55/257ms, 128×128, FOV = 64×64 mm, slice thickness = 10 mm, number of shots = 8, NA = 36, and TR = 2 s. The diffusion gradient was applied perpendicular to the SCs in 32 increments (q_{max} = 0.08 µm^{-1}) and the scan time ~5 hours. Note that these values for δ and Δ violate SGPA. All five SCs were imaged simultaneously. After Fourier transform, a 3D matrix of 32 2D images at various q-values was obtained. An average E(q) was measured in ROIs within the dorsal, ventral, and lateral columns of the SCs (Fig. 1). This average E(q) was fit with Eq. 2 under the constraint $f_E+f_I=1$.

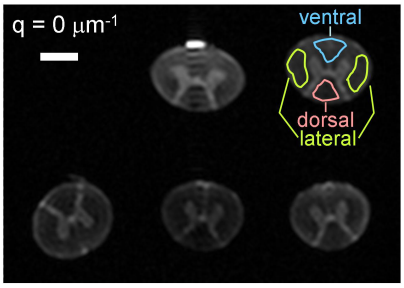

Fig. 1. Sample image at q = 0 µm^{-1}. White bar = 5 mm. ROI locations are shown for the dorsal, ventral and lateral WM columns. The bright spot above the center top SC is residual surface PBS.

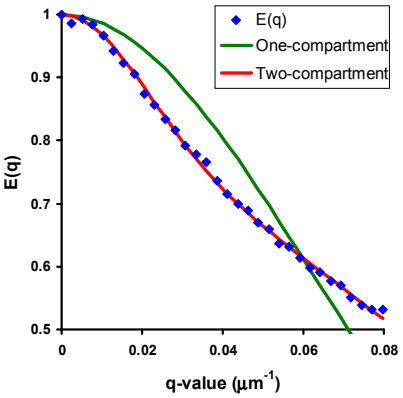

Fig. 2. Sample $E(q)$ for a lateral WM column ROI (blue diamonds) with one-compartment (green line) and two-compartment (red line) fits.

Results and Discussion

Fig. 2 shows a sample E(q) with the fit from Eq. 2. The fit shows good agreement with E(q) (R^2 >0.98). For display purposes, a one-compartment fit (Eq.1) is also shown to illustrate its poor agreement. Fig. 3 shows bar graphs of f_E, Z_E, f_I, and Z_I fitting results for each ROI averaged over all five SCs. Z_I falls within 1-2µm, which is the expected range of axon diameters in mammals[8]. Z_E is lower than that expected for free water (~20µm for Δ = 98.7ms). The ADC calculated from Z_E (~0.25×10^{-3} mm^2/s) agrees with literature values for fixed spinal cord WM tissue[9], which provides further evidence that ADCs measured at low b-values (<2500 s/mm^2) primarily reflect diffusion in ECS[10]. The average f_I was ~0.7, which falls within the expected ICF for WM[11]. An ANOVA analysis indicated no significant differences in f_I among the WM columns as previously seen in mouse SP[5]. Paired t-tests indicated that the dorsal column Z_E and Z_I are significantly smaller than those of the ventral WM column, which matches previous observations of smaller MAD and increased axon density in the dorsal compared with ventral columns[4,8,9].

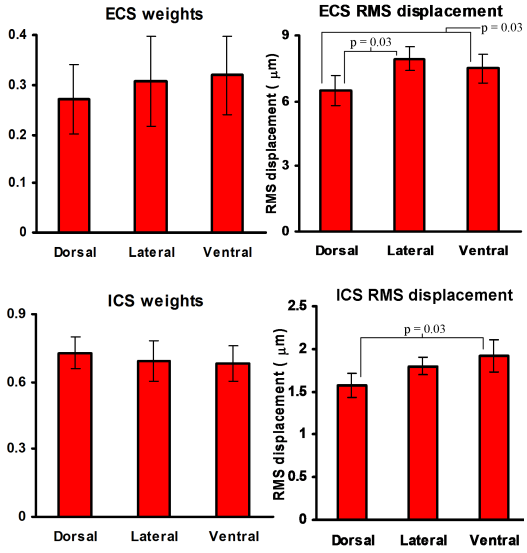

Fig. 3. Bar graphs of f_E (ECS weighting), Z_E (ECS RMS displacement), f_I (ICS weighting), and Z_I (ICS RMS displacement) fitting results Standard deviation bars are shown. Significant p-values (<0.05) of paired t-tests between the different ROIs are shown.

Conclusion

This work demonstrates the feasibility of implementing low q-value diffusion MRI on a 1.5T scanner. The results show that despite violating SGPA, this method has the potential to accurately assess regional axonal architecture with metrics such as MAD and ICF.

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Direct magnetic resonance detection of myelin and prospects for quantitative imaging of myelin density

Michael J. Wilhelm[a,1], Henry H. Ong[a], Suzanne L. Wehrli[b], Cheng Li[a], Ping-Huei Tsai[a,2], David B. Hackney[c], and Felix W. Wehrli[a,3]

[a]Laboratory for Structural NMR Imaging, Department of Radiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; [b]NMR Core Facility, Joseph Stokes Jr. Research Institute, Children's Hospital of Philadelphia, Philadelphia, PA 19104; and [c]Department of Radiology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115

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Magnetic resonance imaging has previously demonstrated its potential for indirectly mapping myelin density, either by relaxometric detection of myelin water or magnetization transfer. Here, we investigated whether myelin can be detected and possibly quantified directly. We identified the spectrum of myelin in the spinal cord in situ as well as in myelin lipids extracted via a sucrose gradient method, and investigated its spectral properties. High-resolution solution NMR spectroscopy showed the extract composition to be in agreement with myelin's known chemical make-up. The 400-MHz ^{1}H spectrum of the myelin extract, at 20 °C (room temperature) and 37 °C, consists of a narrow water resonance superimposed on a broad envelope shifted ∼3.5 ppm upfield, suggestive of long-chain methylene protons. Superimposed on this signal are narrow components resulting from functional groups matching the chemical shifts of the constituents making up myelin lipids. The spectrum could be modeled as a sum of super-Lorentzians with a T_2* distribution covering a wide range of values (0.008–26 ms). Overall, there was a high degree of similarity between the spectral properties of extracted myelin lipids and those found in neural tissue. The normalized difference spectrum had the hallmarks of membrane proteins, not present in the myelin extract. Using 3D radially ramp-sampled proton MRI, with a combination of adiabatic inversion and echo subtraction, the feasibility of direct myelin imaging in situ is demonstrated. Last, the integrated signal from myelin suspensions is shown, both spectroscopically and by imaging, to scale with concentration, suggesting the potential for quantitative determination of myelin density.

myelin in situ | myelin NMR spectrum | super-Lorentzian fitting | ultrashort echo time

Myelin is a critical feature of nervous system white matter (WM) and accounts for 14% of the wet mass of WM (1). It is a lipid–protein bilayer that extends from the outer membrane of glial cells (i.e., oligodendrocytes in the CNS) and discretely winds around individual axonal fibers, leading to an increase in conduction velocity (1). By speeding conduction and reducing axonal energy requirements, myelin makes large and complex organisms possible. Myelin also contributes to the mechanical and functional structure of the axon. In addition, some oligodendrocytic cells and precursors can support action potentials themselves (2). Deficiencies of myelin lay at the core of numerous neurodegenerative disorders, such as multiple sclerosis and schizophrenia (1). These deficiencies may result from developmental or acquired abnormalities in oligodendrocyte function, which also leads to axonal degeneration. Assessment of myelin may reveal CNS abnormalities far beyond those associated with classic demyelinating diseases. MRI of myelin has the potential to characterize not only loss of this important component of the CNS but also to reveal axonal and supporting glial integrity and function.

A diverse assortment of experimental techniques has been applied toward the goal of observing and quantifying myelin. The common methods rely on optical microscopy of histologically stained tissue samples (3). X-ray diffraction (4) and nonlinear optical techniques (5, 6) also provide insight into myelin ultrastructure. Unfortunately, all these techniques are destructive and thus applicable only to animal studies.

More recently, myelin-specific chemical contrast markers that selectively bind to myelin have emerged. Such agents are currently under development for both MRI (7) and positron-emission tomography (8). Although these techniques are potentially promising, concerns over toxicity may pose significant hurdles to their clinical implementation.

So far, MRI has had the greatest impact toward nondestructive myelin assessment in both laboratory animals and humans. Further, MRI has the added benefit that signal contrast originates from endogenous protons and hence is not reliant upon injectable chemical probes nor limited by contrast-related temporal delays.

To date, two indirect MR techniques applicable to studies in vivo have demonstrated histologically correlated sensitivity to myelin: magnetization transfer (MT) and T_2 relaxometry. In MT, cross-relaxation between myelin protons and tissue water is exploited (9). The signal attenuation resulting from off-resonance saturation (MT ratio) has been found to scale with myelin concentration (10). T_2 relaxometry yields T_2 spectra, typically by inversion of the Carr-Purcell echo decay using an inverse Laplace transformation (11). Spectral components with T_2 values ranging from 10 to 50 ms have been assigned to motionally restricted myelin water (12, 13) and have demonstrated strong correlation with myelin-specific histologic staining (12, 14).

Although MT and T_2 relaxometry have shown promise, they both rely on indirect detection of myelin through the interaction of water with myelin. This complex interaction is affected by nonmyelin loss-related changes, which can lead to ambiguities in data interpretation. For example, MT is sensitive not only to myelin content but also to axon density (15). Therefore, even though both techniques may distinguish normal from abnormal WM, they rely on the invariance of the myelin–water interaction.

Direct detection of myelin with MR would remove some complications in the analysis from the intermediate effects of the

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The authors declare no conflict of interest.

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[1]Present address: Department of Chemistry, Temple University, Philadelphia, PA 19122.

[2]Present address: Graduate Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei 10617, Taiwan, Republic of China.

[3]To whom correspondence should be addressed. E-mail: wehrli@mail.med.upenn.edu.

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interaction of water and myelin, and therefore may provide contrast specific to myelin concentration. However, direct detection is complicated by motional restriction of the lipid chains in the myelin bilayer, resulting in broad lines and, consequently, short lifetime of the MR signal.

Perhaps the first characterization of the NMR spectral properties of myelin was by Lecar et al. (16), who studied anhydrous preparations by wide-line proton spectroscopy, concluding that under these conditions the system is in a liquid–crystalline state. To the best of our knowledge, the first report of myelin proton transverse relaxation was by Ramani et al. (17). The authors performed a multiexponential fit of spin-echo decays on fixed human WM samples from normal and multiple sclerosis patients and reported a T_2 value of ~50 μs for myelin protons. They were also able to detect lipid signals by magic-angle spinning proton NMR in slices of normal WM tissue but not in multiple sclerosis lesions. Recently, Horch et al. (18) investigated the T_2^* and T_2 relaxation characteristics of myelin and reported values of ~70 μs as well as a broader distribution ranging from 50 to 1,000 μs.

The transverse relaxation properties of myelin suggest the need for ultrashort echo time (UTE) MRI methods, which entail collection of the free-induction decay immediately after excitation. Typical implementations include either 3D radial sampling with nonselective rf pulses (19) or 2D radial sampling with slice-selective half rf pulses and ramp sampling (20) as previously applied for the characterization of cortical bone matrix and bone water (21, 22).

UTE MRI has been used to image the short T_2^* (i.e., <1 ms) signal from human brain in vivo (23). Unlike applications to study bone, these implementations include long T_2^* suppression methods to attenuate the tissue water signal. Tissue water, because of its rotational mobility and high concentration, has an intense long T_2^* signal that, without suppression, overwhelms signal from short T_2^* components (Fig. S1*A*). Although the images indicated the short T_2^* signal to be predominantly located in WM, no evidence was provided to link this signal to myelin.

In this work we examine the origin and nature of the short T_2^* signal of CNS tissue in freshly excised rat spinal cord (SC) in comparison with purified myelin lipid extract with multinuclear NMR. We further explore the potential for direct detection and quantification of myelin by UTE MRI and discuss the possibilities and technical hurdles associated with translating MRI-based quantification of myelin to the clinic.

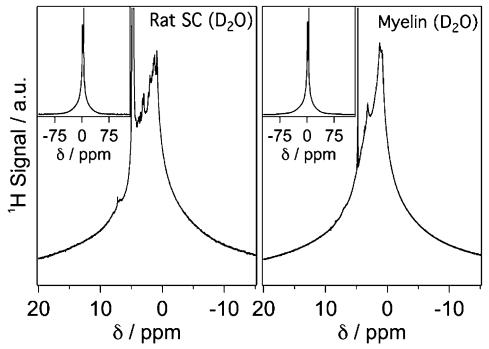

Fig. 1. The ^{1}H NMR spectra from rat thoracic SC after D_2O exchange of tissue water (*Left*) and myelin lipid extract suspended in D_2O (*Right*), showing remarkable similarity. *Insets*: Wide tails present in both spectra. Note that in addition to narrow resonances, likely stemming from proteins, the residual monodeuterium oxide (HDO) resonance is stronger in the tissue spectrum.

Results

High-Resolution ^{1}H NMR Spectra of Intact SC Tissue and Bovine Myelin Extract. Fig. 1 shows a comparison of the ^{1}H NMR spectra collected for a section of excised rat thoracic SC and a deuterium oxide (D_2O) suspension of myelin lipid extract. The SC was first immersed in 99.9% D_2O for 24 h to exchange tissue water with D_2O, and hence attenuate the bulk proton signal (Fig. S1*B*). The protein-free myelin lipid sample was chemically extracted from an intact isolated myelin sample, obtained via a sucrose gradient method (*SI Materials and Methods*) and quantitatively validated with proton-decoupled ^{13}C and ^{31}P NMR spectroscopy (Table 1 and Fig. S2 *A* and *B*).

Both ^{1}H spectra in Fig. 1 show a broad resonance with a superimposed narrow water resonance originating from residual HDO. Although the two broad resonances bear a high degree of similarity, a difference spectrum (i.e., tissue − extract) (Fig. S3) highlights the distinguishing features. First, the superimposed fine structure apparent in the SC spectrum is consistent with mobile proteins as they might occur in the cytoplasm, for example. Second, the difference spectrum contains a broad resonance as it might be expected from membrane proteins. The difference spectrum was generated such that the integrated signal area is 27.9% of the total spectrum, as expected according to the known protein fraction in myelin (details in *SI Results*). Small errors in the difference spectrum could arise because we are ignoring cytoplasmic proteins in this fraction.

UTE MRI of Intact SC. Fig. 2 shows a set of images of freshly excised rat SC, obtained with a 3D radial, ramp-sampled, dual-echo inversion recovery UTE (de-IR-UTE) pulse sequence (Fig. S4). Long T_2^* tissue water signal was attenuated via adiabatic inversion and complex echo subtraction. Adiabatic inversion was used to significantly reduce the signal intensity from tissue water, which would then be further attenuated with echo subtraction. We empirically selected TI to achieve the greatest WM intensity while minimizing gray matter (GM) intensity signal in the complex echo difference image in accordance with the expectation of low signal in GM considering its very low myelin content. Images were collected at both short (20 μs; Fig. 2*A*) and long (1,200 μs; Fig. 2*B*) TE. The magnitude of the complex difference image and signal profile (Fig. 2 *C* and *D*)

Table 1. Lipids of myelin with abbreviations used in the text

Myelin lipid	ID	Molar % Norton*	Molar % NMR†	% labile ^{1}H ± σ‡
Cholesterol	CHOL	44.8	43.1	0.94 ± 0.04
Galactocerebroside	GC	17.5	19.6	2.20 ± 0.39
Galactosulfatide	GS	2.5	NA§	0.28 ± 0.05¶
Phosphatidylethanolamine	PE	3.4	3.9	0.24 ± 0.05
PE plasmalogen	PEpl	11.3	11.8	0.70 ± 0.13
Phosphatidylcholine	PC	8.0	7.8	0.00 ± 0.00
PC plasmalogen	PCpl	0.3	2.0	0.00 ± 0.00
Sphingomyelin	Sph	5.2	5.9	0.37 ± 0.08
Phosphatidylinositol	PI	0.7	2.0	0.19 ± 0.03
Phosphatidylserine	PS	0.2	3.9	0.11 ± 0.01
Total				5.05 ± 0.79

Comparison of average bovine myelin lipid molar ratios reported by Norton et al. (25) and quantitative multi-NMR methods. Also shown are average percentages of labile protons pertaining to each lipid component. PC, phosphatidylcholine; PE, phosphatidylethanolamine.
*From Norton et al. (23).
†Present study, ^{13}C and ^{31}P NMR.
‡Variability (σ, SD) due to lipid chain length [$CH_3(CH_2)_n$; n = 10–25].
§Not measured owing to a lack of an unambiguous resonance.
¶Assuming a GS molar percentage of 2.5%.

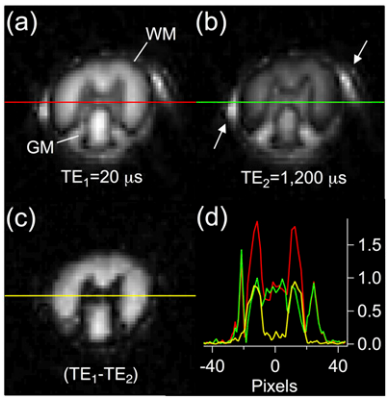

Fig. 2. The 3D de-IR-UTE images from rat thoracic SC averaged over five central slices. Magnitude images obtained for (*A*) TE = 20 μs, (*B*) TE = 1,200 μs, and (*C*) complex difference (*A* − *B*) (maximum-intensity range decreased by a factor of two to highlight myelin signal). (*D*) Intensity profiles across the three images (delineated as red, green, and yellow lines in *A*, *B*, and C, respectively) to show relative WM, GM, and background intensity. The most intense signal, present in the short- and long-echo profiles, originates from residual surface water. WM and GM are indicated in *A*, and arrows highlight residual surface water in *B*. The dark boundary observed at the GM/WM and WM/surface water interfaces in both echo images stems from partial voluming of adjacent regions with different T_1 resulting in destructive interference.

highlight the short T_2^* signal, which predominantly results from myelin protons.

MR Signal Dependence on Myelin Concentration. To separate the myelin and water peaks in the ^{1}H spectra of the myelin-D_2O suspensions, the spectra were modeled as a weighted sum of four super-Lorentzians (SL) for myelin resonances representing protons from general alkyl chain methylenes (noncholesterol, mostly from fatty acid residues), cholesterol alkyl chain methylenes, terminal methyls, and choline, and a Lorentzian for the HDO peak (details in *Materials and Methods*). Fig. 3*A* shows the results from fitting of the proton NMR spectrum of purified myelin suspended in D_2O. The fitting results were virtually identical for all myelin concentrations. Even though the signal envelope is very broad, relatively narrow resonances are also observed, likely due to proton pairs aligned with an average orientation at the magic angle relative to the static field (24).

Relative signal fractions, accounting for losses during excitation and acquisition, along with associated T_2^* distributions of the four SL components, were combined into a myelin T_2^* histogram (Fig. 3*B*). At 20 °C, 26.4% of the total signal has an effective lifetime of <25 μs, 51.8% of <100 μs, and 91.6% of <1,000 μs. At 37 °C these values are 16.9%, 44.8%, and 86.3%, respectively.

Fig. 4*A* shows a series of fitted myelin signals as a function of decreasing myelin concentration. The NMR signal areas for the total and separate spectral components (i.e., HDO and myelin) are plotted in Fig. 4*B*, indicating linear scaling with myelin concentration (R^2 = 0.99). We attribute the positive correlation of the water peak area with myelin concentration as resulting from labile protons from myelin constituents exchanging with D_2O to form HDO. The calculated average percentage of labile protons, for each of the 10 myelin lipid components, is listed in Table 1. The average signal contribution from the 0.1% impurity of the D_2O solvent, calculated as the y-intercept from the line of best fit (Fig. 4*B*), was subtracted from all of the HDO points, yielding an estimate of the labile myelin proton signal contribution. The predicted range of signal contributions from labile protons (5.05% ± 0.79%) agreed well with the experimental HDO peak areas (5.13% ± 2.00%). Given the excess D_2O used

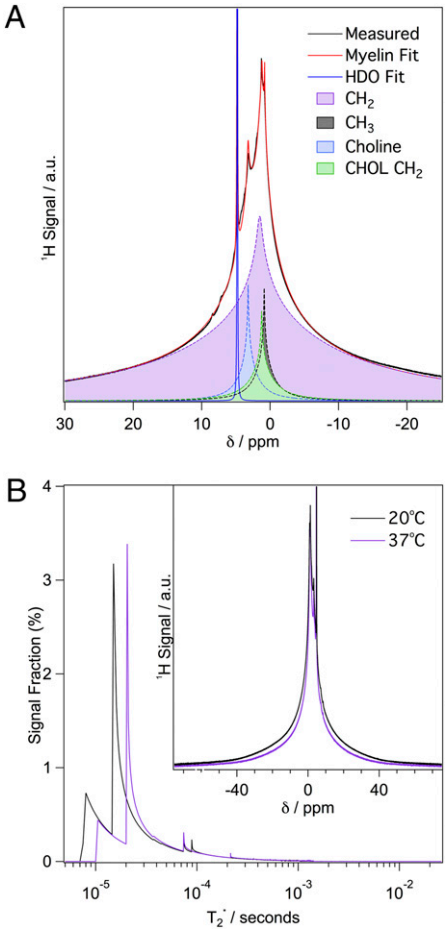

Fig. 3. ^{1}H NMR spectra and analysis of purified bovine myelin extract suspended in D_2O. (*A*) NMR spectrum (black) and SL fitting showing the resulting myelin (red) and HDO (blue) fits, as well as the four individual SL components of myelin (shaded). The four fitted SL components consisted of (*i*) SL containing 74.3% of the intensity, centered at 1.5 ppm, corresponding to the general alkyl chain methylene protons (CH_2), (*ii*) SL containing 12.4% of the intensity, centered at 0.9 ppm, corresponding to the terminal methyl protons (CH_3), (*iii*) SL containing 11.1% of the intensity, centered at 3.2 ppm, corresponding to the choline methyl protons (Choline), and (*iv*) SL containing 2.1% of the intensity, centered at 1.3 ppm, corresponding to the cholesterol alkyl chain methylene protons (CHOL CH_2). (*B*) T_2* histogram of myelin components at 20 and 37 °C derived from the SL fitting. There are small T_2* components that extend up to 26 ms, but these cannot be observed at the displayed scale. *Inset*: Myelin extract spectra collected at the two temperatures.

in the suspensions, it is reasonable to assume that all labile myelin protons had exchanged with deuterium.

Fig. 5 *Inset* shows the complex difference 2D projection de-IR-UTE image for a series of myelin suspensions of increasing concentration. Region of interest (ROI) average intensities from each of the myelin samples in the image are plotted in Fig. 5 and, analogous to the spectral data, are linearly correlated with myelin concentration (R^2 = 0.98).

On the basis of the relaxation characteristics of myelin (notably the lifetime of the various T_2^* components), Bloch equation simulations (details in *SI Materials and Methods*) accounting for losses sustained during the rf pulse sampling suggest that, under the spectral recording conditions, 81.7% of the total spectral signal is recovered, where the shorter T_2^* components account for the majority of the signal lost. The depletion of the imaging signal is more severe because it entails coherence losses during both

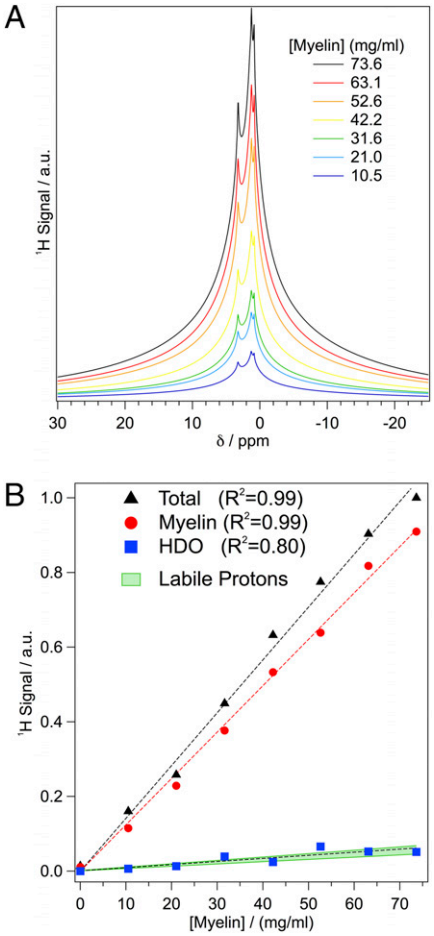

Fig. 4. (*A*) Fitted myelin peaks for myelin suspensions of various concentrations. (*B*) Linear correlation plot of MR signal as a function of myelin concentration for the total (triangle), myelin (circle), and HDO (square) signal components with calculated R^2 values. All two-tailed P values <0.01. Also shown is the calculated signal fraction of labile myelin protons (green shaded).

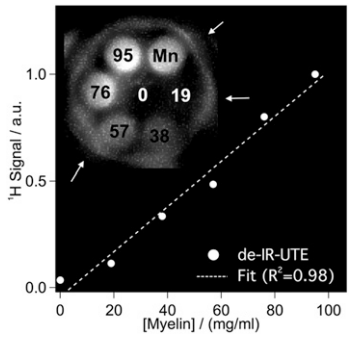

Fig. 5. UTE imaging and analysis of purified bovine myelin extract suspended in D_2O. *Inset*: The 2D projection de-IR-UTE image of six 5-mm NMR tubes filled with myelin/D_2O suspensions of varying concentrations (mg/mL). Mn indicates the tube containing manganese-doped water. Plot of the normalized de-IR-UTE mean ROI intensities vs. myelin concentration (P = 0.0002). Arrows indicate areas of signal from paper towel used to stabilize the tubes.

the adiabatic inversion and excitation pulses, as well as during the period preceding collection of the low-spatial frequencies (TE) and partial saturation. We estimate that with the imaging parameters used in our experiments, 38.0% of the total available myelin proton signal could be recovered.

Discussion

In this work we explored the feasibility of direct imaging and quantification of myelin by magnetic resonance as an alternative to indirect detection techniques such as MT or T_2 relaxometry. Direct detection of myelin would remove any ambiguities in analysis and provide a contrast specific to myelin concentration. To explore the feasibility of direct myelin imaging, we first investigated the origins of the short T_2^* signal in intact SC and myelin extracts. We then presented preliminary UTE with long T_2^* suppression images of myelin in WM.

The purity of our myelin extract was validated by 9.4 T high-resolution proton-decoupled ^{31}P (Fig. S2*A*) and ^{13}C (Fig. S2*B*) NMR, showing good agreement with previously determined molar ratios (25) (Table 1). The slight variations are reasonable given the differences in the anatomical origin of the sample (brain vs. SC) (26), inherent variability of the measurement (27), and the developmentally immature nature of the tissue examined (26). Our ^{13}C spectra also agreed well with Husted et al.'s reported proton-decoupled magic-angle spinning (MAS) ^{13}C NMR spectra of isolated human myelin (28). In that study a synthetic ^{13}C spectrum of myelin was generated as a weighted sum of individual lipid spectra, based upon the known ratios of the individual lipid components. The synthetic spectrum was found to be in good agreement with the observed MAS spectrum, which in turn closely matches our solution spectrum (Fig. S2*B*), except for the absence of signals assigned to protein resonances in the MAS spectrum.

Any conclusions from extracted or synthetically produced myelin beg the question whether the resulting material retains the biophysical properties of native myelin. It is known that in aqueous suspensions myelin lipids spontaneously self-assemble into bilayer structures (i.e., vesicles). In this enthalpy-driven process (29) the myelin lipid suspension regains a structural order reminiscent of physiological myelin minus the protein component.

The spectrum of the isolated, reconstituted myelin exhibited a very broad line with relatively narrow components centered ~3.5 ppm upfield from water, consistent with methylene protons of alkyl chains, the main constituent of myelin. Further, this spectrum bears a high degree of similarity with that of neural tissue, which supports the notion that, upon aqueous suspension of the extract, a bilayer structure analogous to that for native myelin is reconstituted. The SC spectrum is expected to contain nonmyelin contributions such as from proteins. The difference spectrum (Fig. S3) suggests the presence of motionally restricted membrane proteins, along with a small fraction of mobile (perhaps cytoplasmic) proteins yielding narrow resonances.

The myelin resonance is consistent with the SL lineshape of a dipolar-broadened liquid–crystalline lipid system (24, 30) as suggested by several lineshape properties (31), including a small second moment ($M_2 = 1.06 \times 10^8$ s^{-2}), a large fourth moment (M_4) to M_2 ratio [$M_4/(M_2)^2 = 6.57$], and a full width at 1/10 maximum ($\Delta\nu_{1/10}$) to full width at 1/2 maximum ($\Delta\nu_{1/2}$) ratio >3 ($\Delta\nu_{1/10}$: $\Delta\nu_{1/2} = 7$). Following previous work (32), we modeled the myelin lipid extract spectrum as a sum of SLs representing protons from general alkyl chain methylenes, cholesterol alkyl chain methylenes, terminal methyls, and choline. Although the T_2^* distribution showed a wide range of values (0.008–26 ms), the distribution was dominated by that of the alkyl chain methylenes because they make up ~75% of the four myelin lipid proton moieties under consideration. One notes a bimodal distribution (Fig. S5) with the first peak significantly attenuated as expected for an SL lineshape determined by the angular dependence of T_2^* (details in *SI Results*).

In contrast to the SL fitting performed in this work, Horch et al. performed multiexponential fitting of the time-domain signal decay (18). Although the SL lineshape theoretically

cannot be described with a basis set of exponential functions, the authors suggested that the resulting errors would be small for the case of myelin. Horch et al. analyzed free-induction decay signals of myelinated mammalian and amphibian nerves, as well as synthetic myelin at 4.7 T, yielding histograms of relaxation times. The authors detected significant components with T_2^* values of 20 and 70 μs in frog sciatic nerve, which they conjectured to arise from protein and methylene protons of myelin, respectively. In contrast, because our myelin extract was free of protein, the present data alternatively suggest the short T_2^* components (<25 μs) to arise from myelin lipids. This is not to imply that membrane proteins cannot contribute a short T_2^* component, as suggested by the broad resonance in the difference spectrum (Fig. S4) and in other reports (24, 33).

In bovine myelin suspended in D_2O, we found the integrated spectral areas to scale linearly with myelin concentration (Fig. 4), as did ROI intensities of the 2D projection de-IR-UTE images (Fig. 5), thus suggesting that quantitative myelin imaging may be feasible. Direct 3D de-IR-UTE imaging of a rat SC in situ at 400 MHz highlights the potential of such an approach, as demonstrated with images showing signal from the WM regions only. Absolute quantification would require a reference sample, ideally with relaxation and density properties matching myelin. The reference sample should also be chemically stable.

Previously, Waldman et al. (23) obtained images of the human brain using a slice-selective UTE along with soft-tissue suppression, essentially showing intense signal from WM regions of the brain, which they attributed to short T_2^* components. Under these conditions [i.e., selective half-*sinc* pulses of 400- to 600-μs duration (34)], all but the longest T_2^* components of the myelin protons would elude detection.

Our results indicate approximately 20% of the protons in myelin lipids to have an effective T_2^* less than 25 μs. Even under the more favorable conditions of our imaging experiments, the majority of these short T_2^* components remains undetectable. The very short lifetime of the signal has potentially adverse effects on the point-spread function (PSF) manifesting as blurring. A simple estimation based on the FWHM of the spectra (Fig. 3) predicts an intrinsic resolution (defined as the minimum achievable pixel size) on the order of 100 μm, which is approximately one pixel with our current imaging parameters (more details in *SI Materials and Methods*). It should be noted that although the blurring from the shortest T_2^* component would be greater, its impact on the PSF is negligible because this signal fraction remains virtually undetected.

A limitation that needs to be noted for this method is that it detects myelin solely on the basis of its T_2^* properties. Thus, errors in long T_2^* suppression may lead to signal misclassified as short T_2^* and hence falsely identified as myelin. Such errors could be accounted for and perhaps mitigated by tailoring a reference sample so as to contain water of comparable concentration and relaxation times to those of neural tissue. Last, there are other possible nonmyelin short T_2^* sources that could contribute to the UTE image intensity, including glial cell membranes, calcifications, tissue scars, vasculature, and hemorrhage (23), that would be indistinguishable from myelin.

Another potential problem could arise from saturation of the myelin signal via cross-relaxation (35). Even though adiabatic inversion of tissue water has minimal direct effect on the myelin lipid proton signal, transfer of magnetization from the water to the myelin proton pool could occur via chemical exchange or dipolar coupling. This mechanism would result in a reduction in the detected myelin signal, an effect that requires further scrutiny.

The potential for translation of the method to the clinic will be challenging. Nevertheless, it is encouraging to note that with dedicated hardware rf pulses of 20 μs or less have already been shown to be feasible on clinical equipment at 3 T field strength, as in recent work by Wu et al., who imaged the collagen matrix of cortical bone (36). Further, at 37 °C the measured T_2^* values increase by *ca.* 30% (Fig. 3*B*), raising the minimum T_2^* value from 8 to 10.5 μs. At body temperature the scan parameters used in our current experiments at 9.4 T (except for a 20-μs hard pulse of 7.6° flip angle to match the peak B_1 amplitudes of clinical head coils) predict 4.9% of the total myelin proton signal [i.e., 0.7% of the total proton signal given that myelin accounts for 14% of WM (1)] to be recoverable on a 3 T clinical MRI system (for calculation details see *SI Materials and Methods*).

Given that tissue proton concentration is ∼100 M, the concentration of detectable myelin protons is approximately 700 mM. In comparison with proton MR spectroscopic imaging (MRSI) of brain metabolites, where the metabolite concentrations are on the order of 10 mM, detectable myelin proton concentration, and hence intrinsic SNR, should be a factor of approximately 70 greater than that of typical metabolites. However, this gain in SNR compared with MRSI is mitigated by the reduced sampling time imposed by the much shorter T_2^* of the protons in myelin compared with those in brain metabolites. We estimate reduced overall sampling time to result in a loss on the order of a factor of 10. Given a reported resolution for MRSI of 5–10 mm (37), we project the resolution achievable with our method to be roughly on the order of 2.5–5 mm with T_2^*-induced PSF blurring not exceeding 0.6 mm (*SI Results*).

Conclusions

We have characterized the spectral properties of the myelin proton signal in situ, as well as in reconstituted suspensions of myelin lipid extract. Our results show that the short T_2^* component of WM originates primarily from myelin lipid protons and further that direct imaging of these protons is possible even though the shortest components are not detectable. Last, our analysis suggests that translation from the laboratory to clinical MRI will be challenging.

Materials and Methods

All MR spectroscopy and imaging experiments were performed on a 9.4 T vertical-bore spectrometer/microimaging system (Bruker DMX 400) with Micro2.5 gradients (100 G/cm maximum strength) and BAFPA40 amplifiers.

Neural Tissue Preparation. SC samples were harvested from healthy adult Sprague-Dawley rats (Charles River Laboratories) and bovine spinal columns (Bierig Brothers Veal and Lamb Products). The rats were killed by carbon dioxide asphyxiation in accordance with an Institutional Animal Care and Use Committee-approved protocol. After killing, rat spinal columns were removed, and the SC was dissected out.

NMR Spectroscopy. High-resolution proton-decoupled ^{13}C NMR [Sweep width (SW) = 24 kHz, number of scans (NS) = 36,768, number of real and imaginary data points (TD) = 65,536, repetition time (TR) = 1.36 s, α = 30°] and proton-decoupled ^{31}P (SW = 3.23 kHz, NS = 8,536, TD = 8,192, TR = 1.27 s, α = 30°) spectra were collected for samples of purified bovine myelin extract, dissolved in a (5:4:2) ternary mixture of deuterated chloroform (99.8 atom % D; Acros Organics), methanol (99.8 atom % D; Acros Organics), and 0.2 M EDTA/water (99.9 atom % D; Sigma-Aldrich).

All ^1H NMR spectra were collected with the following parameters: SW = 100 kHz, NS = 256, TD = 262,144, TR = 3.6 s, α = 90°, pulse duration = 9.6 μs. Freshly excised SC sections (<2-h postmortem interval) were immersed in a perfluorinated oil (Fomblin-Y; Sigma-Aldrich) before experiments.

SL Fitting of Proton Spectrum. As described by Wennerström (30), only partial averaging of dipolar coupling via translational and rotational diffusion occurs, resulting in a dipolar-broadened liquid–crystalline lipid system with an SL lineshape that can be written as:

$$L(\omega) = \int_0^{\pi/2} \frac{\sin(\theta)}{\left|3\cos^2(\theta)-1\right|} f\left[\frac{\omega-\omega_0}{\left|3\cos^2(\theta)-1\right|}\right] d\theta \qquad [1]$$

where ω_0 is the chemical shift, θ is the angle of the lipid bilayer surface normal with respect to B_0, and $f(\omega\text{-}\omega_0)$ is any highly peaked lineshape such as

a Gaussian or Lorentzian. Assuming θ is uniformly sampled and setting $f(\omega-\omega_0)$ to be a Lorentzian, it can be seen from Eq. **1** that an SL is composed of a series of scaled Lorentzians. From the widths and intensities of these Lorentzians, the T_2* distribution of a single SL can be calculated. Protons at different chemical shifts (e.g., alkyl chain methylenes, terminal methyls, and choline) are each expected to give rise to SL lineshapes (32).

Spectral fitting was performed in Matlab (Mathworks). Four SLs were used to represent general alkyl chain methylenes (noncholesterol, mostly from fatty acid residues), cholesterol alkyl chain methylenes, terminal methyls, and choline, whereas a single Lorentzian was used to model residual HDO. Because cholesterol alkyl chain methylenes sit deep within the lipid bilayer, it is reasonable to expect them to be more mobile than the general alkyl chain methylenes, therefore resulting in a narrower SL. The chemical shifts of each SL were set to the known isotropic shift of the various moieties, and the width and relative intensities were free parameters. The R^2 of the fit was greater than 0.99.

UTE MR Imaging. The 3D de-IR-UTE imaging (Fig. S4): SW = 200 kHz, TE = 20/1,200 μs, TI = 500 ms, TR = 1 s, field of view = 15 mm, matrix size = 128 × 128 × 128, number of views = 5,342, pulse duration = 20 μs. The sequence was based on that used by Anumula et al. (38). TI was determined empirically as the duration yielding optimal GM suppression (because GM is expected to have negligible myelin concentration) in a complex difference image. A refocusing gradient was applied immediately after the first readout gradient, after which a second gradient echo was collected at TE = 1,200 μs. A 3D image of the short T_2* components was obtained as the complex difference of the two echo images (i.e., $TE_1 - TE_2$). A complex difference is necessary to distinguish the possible presence of both inverted and non-inverted voxel signals.

A 2D projection de-IR-UTE sequence was used to image the series of myelin/D_2O suspensions to avoid signal losses resulting from settling of myelin during scanning. The Mn doped water phantom served to identify the locations of the samples in the image. All experimental parameters were identical to those used in the 3D de-IR-UTE experiments.

All image reconstruction was done in Matlab (Mathworks) using a fast gridding algorithm (39) and incorporating k-space trajectory correction (40). All images were smoothed via bilinear interpolation with Image J (National Institutes of Health).

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Super-Lorentzian framework for investigation of T_2^* distribution in myelin

Michael J. Wilhelm[1], Henry H. Ong[2], and Felix W. Wehrli[2]

[1]*Department of Chemistry, Temple University, Philadelphia, PA, United States,* [2]*Laboratory for Structural NMR Imaging, Department of Radiology, University of Pennsylvania School of Medicine, Philadelphia, PA, United States*

Introduction

Deficiencies of myelin, a lipid bilayer sheath critical for normal function of white matter (WM), lay at the core of numerous neurodegenerative disorders such as multiple sclerosis and schizophrenia (1). At present, there are few alternatives to destructive histologic methods to directly assess myelin. The short T_2^* of myelin protons make ultra-short echo time (UTE) MRI a potential imaging modality to directly detect myelin (2). In contrast, indirect MRI methods such as magnetization transfer and T_2 relaxometry are based on complex interactions between water and myelin, which can lead to ambiguities in data analysis. Characterizing the T_2^* distribution of myelin is key to developing optimal UTE methods for myelin imaging. Previous attempts have used multi-exponential fitting of the FID (3), which is not only an ill-posed problem (4), but also theoretically incorrect. Myelin is a liquid crystalline lipid system that is described by a sum of super-Lorentzians (SL) rather than a multi-Lorentzian lineshape (5, 6). Here, we use this SL framework to calculate T_2^* distributions by fitting ^{1}H NMR spectra of myelin lipid extract and intact rat spinal cord (SC).

Theory and Methods

According to Wennerström (6), due to averaging effects from translational and rotational diffusion, for a given orientation of a lipid bilayer, the lineshape can be expressed as: $L(\delta\text{-}\delta_0, \theta) = |3\cos^2\theta - 1|^{-1} f[(\delta\text{-}\delta_0)/|3\cos^2\theta - 1|]$ (Eq.1), where δ is the chemical shift centered at δ_0, θ is the angle of the lipid bilayer surface normal with B_0, and $f(x)$ is any highly peaked lineshape such as a Gaussian or Lorentzian. The SL lineshape, $L_{SL}(\delta\text{-}\delta_0)$, results from a uniform sampling of θ from 0 to $\pi/2$: $L_{SL}(\delta\text{-}\delta_0) = \int L(\delta\text{-}\delta_0, \theta)\sin\theta\, d\theta$ (Eq.2). By assuming $f(x)$ to be a Lorentzian, it can be seen from Eqs. 1 and 2 that a SL is composed of a series of scaled Lorentzians. From the widths and intensities of these Lorentzians, the T_2^* distribution of a single SL can be calculated. Multiple SLs have been used to fit NMR spectra of model membrane systems in which the SLs arise from protons at different chemical shifts, e.g. alkyl methylenes, terminal methyls, and choline (7). Therefore, it is possible in theory to perform a multi-SL fit of a ^{1}H NMR spectrum of myelin and calculate a T_2^* distribution.

Rat and bovine SC samples were harvested from Sprague-Dawley rats (Charles River Labs) and a local butcher. Myelin lipids were extracted from bovine SC tissue with a sucrose gradient method (8), dissolution in a ternary mixture (chloroform/methanol/water), and lyophilization. Previous work has shown that this protocol extracts myelin lipids with little to no protein (2). Dehydrated myelin lipid extract was then re-suspended in 99.9% D_2O (Sigma-Aldrich) to regenerate a bilayer structure. ^{1}H NMR spectra at 9.4T (DMX-400, Bruker Instruments) were obtained for a freshly excised rat thoracic SC immersed in Fomblin (Sigma-Aldrich), as well as the myelin lipid extract. Rat SC was immersed in D_2O for 24 hrs prior to experiments to reduce the dominant tissue water peak.

Spectral fitting was performed in Matlab (Mathworks). Four SLs were used to represent general alkyl chain methylenes, cholesterol alkyl chain methylenes (as they have shorter chain lengths), terminal methyls, and choline, while a single Lorentzian was used to model residual HDO. The chemical shifts of each SL were set to the known isotropic shift of the various moieties, and the width and relative intensities were free parameters.

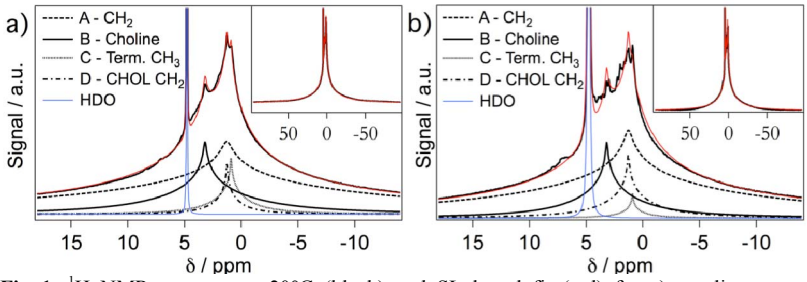

Fig 1. ^{1}H NMR spectra at ~20°C (black) and SL based fit (red) for a) myelin extract (R^2=0.999) and b) rat SC (R^2=0.997). The four SL and Lorentzian peaks of the fit are shown. Full spectral width is shown in inset with HDO peak truncated.

Results and Discussion

Fig. 1 shows myelin lipid extract and rat SC ^{1}H NMR spectra with the results of the four-component SL fits. Both spectra are comprised of a narrow peak from residual HDO and a broad resonance (linewidth ca. 1700Hz) from myelin. A large 4th to 2nd moment ratio (ca. 6.6), suggests that this broad resonance has a SL lineshape. The SC ^{1}H NMR spectrum had additional minor peaks from intracellular proteins and other non-myelin protons. Despite the additional complexity of the SC spectrum, both SL fits agree well with the ^{1}H NMR spectra ($R^2 > 0.99$). Fig. 2 shows the relative fractions (theoretical and fitted) of the four SL myelin components in myelin extract and intact rat SC. As expected, the signal is dominated (>70%) by alkyl methylene protons. Deviations from theoretical values may result from inaccuracies of the SL framework to describe non-chain alkyl protons, e.g. choline and terminal methyls.

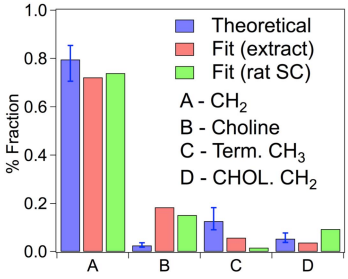

Fig 2. Relative fraction fitting results of the four SL components with expected theoretical fractions. Error bars account for variation in alkyl chain length.

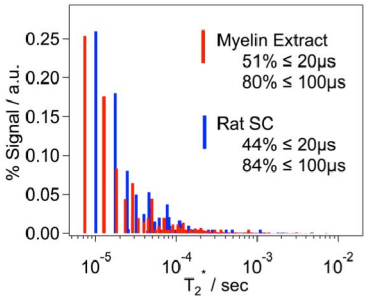

Fig 3. Calculated T_2* distributions for myelin lipid extract (red) and rat SC (blue). Signal fractions with T_2^* <20 and <100 μs are reported.

Fig. 3 shows the T_2^* distributions derived from the SL fits for the myelin lipid extract and SC. The T_2^* distributions are highly skewed with a wide range (10μs to 10ms). Despite this range, roughly 50% (80%) of the signal has a T_2^* less than 20μs (100μs). This result highlights the difficulty of direct myelin imaging even with UTE MRI. Further investigation is needed to study the system at body temperature as increased molecular motion is likely to result in longer effective T_2^*s.

Conclusion

This work uses a SL framework to characterize the T_2^* distribution of myelin, which would provide guidance toward developing UTE methods for myelin imaging. The results indicate that at ambient temperature ~50% of the myelin lipid proton signal has $T_2^* < 20$ μs.

References: 1. van der Knaap, MS et al, *Magnetic Resonance of Myelin, Myelination, and Myelin Disorders*, Springer-Verlag (1995). 2. Wilhelm, MJ et al, *Proc. of the ISMRM* (2011). 3. Horch et al, *Mag. Res. Med.*, **66**:24 (2011). 4. Epstein, CL et al, *SIAM Rev.*, **50**:504 (2008). 5. Bloom, M et al, *Chem. Phys. Lipids*, **4**:107 (1975). 6. Wennerstroem, H et al, *Chem. Phys. Lett.*, **18**:41 (1973). 7. Ulmius, J et al, *Biochim. Biophys Acta*, **389**:197 (1975). 8. Larocca, JN et al, *Curr. Protoc. Cell Biol.*, **3.25**:1 (2006). **Acknowledgements:** NIH T32 EB00814